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PRINCIPAL INVESTIGATOR: Yongmun Choi
Motonari Uesugi

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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6. AUTHOR(S)

Yongmun Choi
Motonari Uesugi

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

E-Mail: ychoi@bcm.tmc.edu

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13. ABSTRACT (Maximum 200 Words)

Overexpression of the Her2 protein has been found in ~30% of breast tumors, and the inhibition of Her2 expression may be an effective way to treat Her2-positive patients. In the first year, the P.I. and co-workers discovered adamanolol, a drug-like compound that impairs the viability of Her2 positive breast cancer cell lines by reducing the expression of Her2. In the second year of funding, we conducted structure-activity relationship studies of adamanolol derivatives and designed a second-generation compound we named "wrenchnolol." The wrench-shaped structure of wrenchnolol permitted detailed biochemical and biophysical analysis of the compound including evaluatiuon of dissociation constants, subcellular localization, binding selectivity in cells. Wrenchnolol is a chemically tractable compound that is suited for mechanistic analysis and for further application.

14. SUBJECT TERMS

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Introduction

Her2 is considered a major target for breast cancer, because overexpression of Her2 is associated with poor overall survival and enhances malignancy and the metastatic phenotypes in breast cancers.[1-5] In a previous annual summary, the P.I. and co-workers reported identification of chemical inhibitor of Her2 transcription by cell-based screening of chemical libraries.[6] The compound that we call adamanolol inhibits Her2 transcription by disrupting the interaction of two cancer-linked nuclear proteins, ESX and Sur-2 (DRIP130). The drug-like molecule showed strong cytotoxicity against Her2-overexpressing breast cancer cells, but had much milder effects on MDA-MB468 with no detectable levels of Her2. Thus, adamanolol could serve as a drug lead for breast cancer therapy. Detailed analysis of unique inhibitors of protein-protein interactions provides a basis for the future endeavor in the challenging field of drug discovery. In the second year of funding, the P.I. analyzed the structure-activity relationship of adamanolol derivatives and designed a second-generation compound we named wrenchnolol.

Body

Structure-activity relationship in cells and in vitro

Structure-activity relationship of adamanolol derivatives is summarized in Table 1 (see Table 1 in attached paper). In one group of derivatives (the R1 substituents in Table 1), the indole ring of adamanolol was substituted. Replacement with a methyl group (12a) abolished the ability of adamanolol to kill Her2-positive SK-BR3 breast cancer cells (Table 1) and to block the interaction of ESX with Sur-2 *in vitro* (see Fig. 1 in the attached paper), while the derivatives with indole-like pharmacores (5a-f, 12b) retained the biological activity (Table 1). Introduction of an arylsulfonyl group, especially a tosyl group (5a), at the *N*1 position of the adamanolol indole ring increased the potency in cells and Sur-2-binding activity *in vitro*, reminiscent of the improved 5-HT receptor-binding activity of *N*-arylsulfonyltryptamines.[7] Although full optimization of the R1 group is still only partly achieved, the observed importance of the indole-mimicking structures is in good agreement with the reported contribution of a tryptophan residue to the specific interaction of ESX with Sur-2.

In another series of derivatives (the R3 substituents in Table 1), the adamantane group of adamanolol was substituted (11a-d). Replacement either with thienyl (11c), tolyl (11d), or methyl (11a) groups resulted in significant loss of the biological activity (Table 1), and the derivative with a

methyl group (11a) exhibited almost no ability to block the ESX-Sur-2 interaction in vitro (see Fig. 1 in the attached paper). The only substitutent that retained a comparable level of activity was a biphenyl group (11b), a chemical module often used to mimic nonpolar amino acids.[8] The bulky, hydrophobic adamantane group may mimic the cluster of isoleucines and leucines on the face of the ESX helix and perhaps participates in binding to the hydrophobic pocket in Sur-2.

The isopropyl group extended from the urea junction of adamanolol was also replaced by a range of bulky substituents (the R2 substituents in Table 1). These derivatives (4a-c, e) had biological activity comparable to that of adamanolol in cells and inhibited the ESX-Sur-2 interaction in vitro as much as adamanolol. By contrast, simple removal of the isopropyl group (4d) abolished both the biological and Sur-2 binding activity. The analogous consequences of all the bulky substituents and the complete loss of the activity in 4d highlight the importance of the bulkiness of the R2 substituents for activity. A bulky substituent at the R2 position enforces the s-cis configuration around the urea linker, which may bring the indole ring and the adamantane group into close proximity and could form a helix-like nonpolar surface for the interaction with Sur-2.

Design of "wrenchnolol"

The structure-activity relationship of adamanolol was translated into the design of the second-generation compound that we named wrenchnolol (see Fig. 2A in the attached paper). As expected, the wrench-shaped, water-soluble molecule inhibited the ESX-Sur-2 interaction in vitro more strongly than adamanolol, presumably due to the presence of its N-tosyl group (Fig. 1), and was no less active than adamanolol in killing SK-BR3 cells (IC50 = 6.9 μM) despite its increased hydrophilicity (Table 1). Wrenchnolol impaired the ability of the ESX activation domain to stimulate the transcription of a reporter gene in cells, whereas it had much milder effects on those of the activation domains of VP16 and NF-κB p65, two functionally irrelevant activation domains structurally similar to the ESX activation domain (Fig. 2B). In contrast, compound 4d, which exhibited little activity in killing SK-BR3 cells, had almost no effects on the ESX activation domain (Fig. 2B). Western blot analysis of wrenchnolol-treated SK-BR3 cells showed that wrenchnolol (7b) reduces the expression of the Her2 protein in cells, but not that of α-tubulin (Fig. 2C). Although expression of other genes may be influenced by wrenchnolol, these cellular effects are consistent with its inhibition of the ESX-Sur-2 interaction in vitro.

Key research accomplishments

1. Examination of the structure-activity relationship of adamanolol derivatives.

2. Desigh of wrenchnolol.

Reportable outcomes

1. Asada, S., **Choi, Y.**, and Uesugi, M. (2003) A gene-expression inhibitor that targets an α-helix-mediated protein interaction. *J. Am. Chem. Soc.* **125**, 4992-4993

2. Shimogawa, H., Kwon, Y., Mao, Q., Kawazoe, Y., **Choi, Y.**, Asada, S., Kigoshi, H., and Uesugi, M. (2004) A wrench-shaped synthetic molecule that modulates a transcription factor-coactivator interaction. *J. Am. Chem. Soc.* **126**, 3461-3471

Conclusion

The structure-activity relationship of adamanolol derivatives was used to gain insight into how it disrupts the interaction of the ESX activation domain with Sur-2. The information gained from these experiments was translated into the design of wrenchnolol, a second-generation compound of adamanolol whose wrench-like structure permitted further biochemical and biophysical analysis. Wrenchnolol is a chemically tractable compound that is suited for mechanistic analysis and for further studies including animal studies.

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Appendix: attached

J|A|C|S
ARTICLES
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A Wrench-Shaped Synthetic Molecule that Modulates a Transcription Factor−Coactivator Interaction

Hiroki Shimogawa,[†,‡] Youngjoo Kwon,[†] Qian Mao,[†] Yoshinori Kawazoe,[†] Yongmun Choi,[†] Shinichi Asada,[†] Hideo Kigoshi,[*,‡] and Motonari Uesugi[*,†]

Contribution from The Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, and The Department of Chemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8571, Japan

Received October 3, 2003; E-mail: muesugi@bcm.tmc.edu; kigoshi@chem.tsukuba.ac.jp

Abstract: Development of synthetic molecules that provide external control over the transcription of a given gene represents a challenge in medicinal and bioorganic chemistry. Here we report design and analysis of wrenchnolol, a wrench-shaped synthetic molecule that impairs the transcription of the *Her2* oncogene by disrupting association of transcription factor ESX with its coactivator Sur-2. The "jaw" part of the compound mimics the α-helical interface of the activation domain of ESX, and the "handle" region accepts chemical modifications for a range of analysis. A water-soluble handle permitted NMR study in aqueous solution; a biotinylated handle verified the selectivity of the interaction, and a fluorescent handle confirmed the cell permeability of the compound. The case study of wrenchnolol foreshadows the promise and the challenge of targeting protein−protein interactions in the nucleus and may lead to the development of unique synthetic modulators of gene transcription.

Introduction

Regulation of gene expression by transcription factors touches many aspects of eukaryotic biology. The biological importance of transcriptional regulation has fueled efforts aimed at discovering means of controlling gene transcription by synthetic molecules. Small-molecule ligands of nuclear receptor transcription factors are among the most successful examples of such molecules and have led to the development of a number of pharmaceuticals.[1,2] Inhibitors of histone deacetylases that modulate gene transcription have also proven to be useful in treating certain types of human diseases.[3] Furthermore, approaches that address the interactions between DNA and transcription factors have been tested, exemplified by the design of synthetic polyamide molecules that display sequence-specific DNA binding at nanomolar concentrations.[4]

An alternative approach to controlling gene transcription is disruption or manipulation of the interaction between a transcription factor and a so-called coactivator protein. Physical association with a coactivator enables a DNA-binding transcription factor to stimulate transcription by recruiting the components of transcriptional machinery, including chromatin-remodeling proteins, to specific promoters.[5,6] Pharmacological intervention in these interactions would modulate gene transcription and might serve as a potential therapeutic strategy. Although inhibiting protein−protein interactions with small molecules is generally difficult, direct contacts of a transcription factor with its coactivators are often mediated by a short α-helical segment of the activation domain,[7−12] suggesting that inhibition by small organic molecules is a reasonably tractable problem.

A good example may be the association between the activation domain of ESX transcription factor (ESE-1/ELF3/ERT/Jen) and its coactivator Sur-2 (a Ras-linked subunit of the human mediator complex).[13] ESX is an epithelial-specific transcription factor that activates *Her2*, an oncogene whose overexpression occurs in ~30% of breast cancer patients.[14,15] ESX binds and strongly activates the *Her2* promoter,[16] and the ESX-binding site in the *Her2* promoter is required for high-level expression of *Her2* in breast cancer cells.[17] The interaction

[†] Baylor College of Medicine.
[‡] University of Tsukuba.

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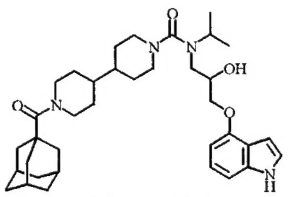
Chart 1



Adamanolol

of ESX with Sur-2 is mediated by one face of an eight-amino acid α-helical region in the ESX activation domain (Ser-Trp-Ile-Ile-Glu-Leu-Leu-Glu), and the tryptophan residue in the hydrophobic face of the helix makes a unique contribution to the specificity of the interaction.[13] A small-molecule inhibitor of the interaction has been discovered by screening a chemical library enriched in indole-mimicking π-electron-rich pharmacores and named adamanolol (Chart 1).[18] The drug-like compound inhibits the ability of the ESX activation domain to stimulate transcription in cells, blocks the interaction of ESX with Sur-2 in vitro, represses the expression of *Her2* gene in cells, and impairs the viability of *Her2*-positive breast cancer cell lines. Adamanolol is a unique synthetic molecule that controls gene expression by modulating a transcription factor–coactivator interaction.

Detailed analysis of unique inhibitors of protein–protein interactions provides a basis for the future endeavor in the challenging field of drug discovery. Here we describe structure–activity relationships of adamanolol derivatives that were used to gain insight into how it disrupts the interaction of the ESX activation domain with Sur-2. The information gained from these experiments was translated into the design of wrenchnolol, a second-generation compound of adamanolol whose wrench-like structure permitted further biochemical and biophysical analysis. Wrenchnolol may also serve as a starting point for designing unique synthetic modulators of gene transcription.

Results

Synthesis of Adamanolol and Its Derivatives. The synthesis of adamanolol and its derivatives is summarized in Scheme 1. Coupling of 4-hydroxyindole and epichlorohydrin afforded glycidyl 4-indolyl ether (**1a**) quantitatively. The ether **1a** was reacted with isobutylamine, *tert*-butylamine, ammonia, or mono-*N*-Boc-1,3-propanediamine to give the corresponding amines **2a**–**d**. Pindolol, amines **2a**–**d**, propranolol, *N*-(3-methoxy-2-hydroxypropyl)isopropylamine, or *N*-[3-(1-naphthyloxy)-2-hydroxypropyl]isopropylamine were then coupled with amide **3** (see below) through the conversion of **3** to an imidazolide and its N-activation by methyl iodide. The coupling resulted in the formation of a urea linkage to afford adamanolol and its analogues **4a**–**e** and **12a**–**b**. The sulfonyl analogues of adamanolol (**5a**–**f**) were prepared by reacting adamanolol with the corresponding sulfonyl chlorides.

The amide **3** was obtained by coupling adamantanecarbonyl chloride with 4,4′-bipiperidine. Reaction of acetic anhydride or 4-biphenylcarbonyl chloride instead of adamantanecarbonyl chloride yielded amides **10a** and **10b**, which were converted to **11a** and **11b**, respectively.

Structure–Activity Relationship in Cells and in Vitro. The structure–activity relationship of adamanolol derivatives is summarized in Table 1. In one group of derivatives (the R^1 substituents in Table 1), the indole ring of adamanolol was substituted. Replacement with a methyl group (**12a**) abolished the ability of adamanolol to kill *Her2*-positive SK-BR3 breast cancer cells (Table 1) and block the interaction of ESX with Sur-2 in vitro (Figure 1), while the derivatives with indole-like pharmacores (**5a**–**f**, **12b**) retained the biological activity (Table 1). Introduction of an arylsulfonyl group, especially a tosyl group (**5a**), at the N1 position of the adamanolol indole ring increased the potency in cells and Sur-2 binding activity in vitro, reminiscent of the improved 5-HT receptor-binding activity of *N*-arylsulfonyltryptamines.[19] Although full optimization of the R^1 group is still only partly achieved, the observed importance of the indole-mimicking structures is in good agreement with the reported contribution of a tryptophan residue to the specific interaction of ESX with Sur-2.

In another series of derivatives (the R^3 substituents in Table 1), the adamantane group of adamanolol was substituted (**11a**–**d**). Replacement either with thienyl (**11c**), tolyl (**11d**), or methyl (**11a**) groups resulted in significant loss of the biological activity (Table 1), and the derivative with a methyl group (**11a**) exhibited almost no ability to block the ESX–Sur-2 interaction in vitro (Figure 1). The only substitutent that retained a comparable level of activity was a biphenyl group (**11b**), a chemical module often used to mimic nonpolar amino acids.[20–22] The bulky, hydrophobic adamantane group may mimic the cluster of isoleucines and leucines on the face of the ESX helix and perhaps participates in binding to the hydrophobic pocket in Sur-2.

The isopropyl group extended from the urea junction of adamanolol was also replaced by a range of bulky substituents (the R^2 substituents in Table 1). These derivatives (**4a**–**c**,**e**) had biological activity comparable to that of adamanolol in cells and inhibited the ESX–Sur-2 interaction in vitro as much as adamanolol. By contrast, simple removal of the isopropyl group (**4d**) abolished both the biological and Sur-2 binding activity. The analogous consequences of all the bulky substituents and the complete loss of the activity in **4d** highlight the importance of the bulkiness of the R^2 substituents for activity. A bulky substituent at the R^2 position enforces the s-cis configuration around the urea linker, which may bring the indole ring and the adamantane group into close proximity and could form a helix-like nonpolar surface for the interaction with Sur-2.

Design of "Wrenchnolol". The structure–activity relationship of adamanolol was translated into the design of the second-generation compound that we named wrenchnolol (Figure 2A). As expected, the wrench-shaped, water-soluble molecule inhibited the ESX–Sur-2 interaction in vitro more strongly than adamanolol, presumably due to the presence of its *N*-tosyl group (Figure 1), and was no less active than adamanolol in killing SK-BR3 cells (IC_{50} = 6.9 μM) despite its increased hydrophilicity (Table 1). Wrenchnolol impaired the ability of the ESX

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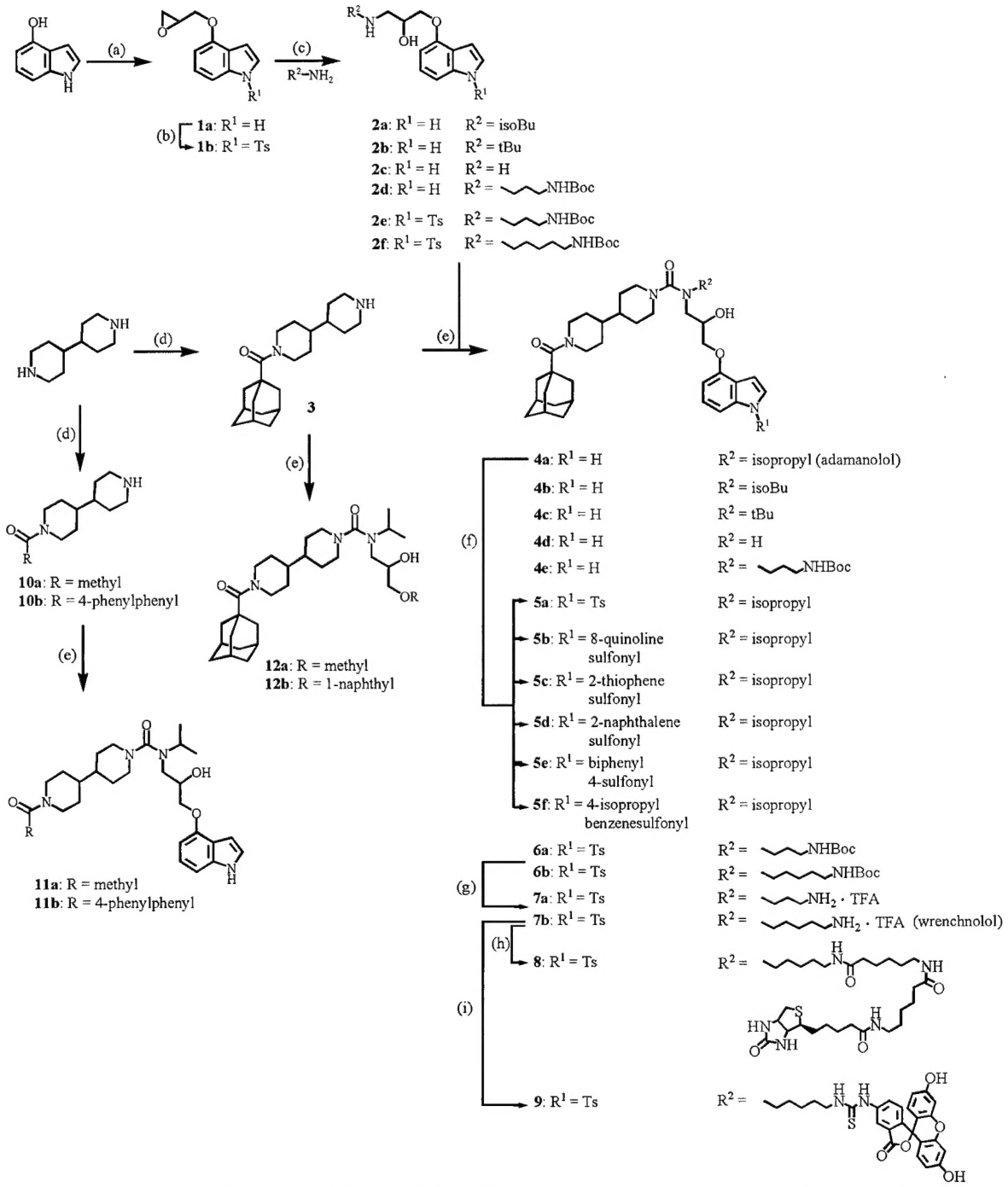
Scheme 1 [a]

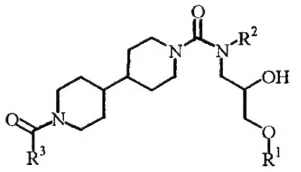


[a] Conditions: (a) Epichlorohydrin, KOH, DMSO, 45 °C. (b) NaH, TsCl, THF, rt. (c) Isobutylamine, *tert*-butylamine, NH_3, mono-*N*-Boc-1,3-propanediamine, or mono-*N*-Boc-1,5-pentanediamine, MeOH, 60 °C. (d) Adamantanecarbonyl chloride, acetic anhydride, or 4-biphenylcarbonyl chloride, Et_3N, $CHCl_3$, reflux. (e) (1) CDI, THF, reflux; (2) CH_3I, CH_3CN, rt; (3) amine **2a**−**f**, pindolol, *N*-(3-methoxy-2-hydroxypropyl)isopropylamine, or propranolol, Et_3N, CH_2Cl_2, rt. (f) NaH, TsCl, THF, rt. (g) TFA, $CHCl_3$, rt. (h) Biotin-XX-NHS, DCC, DMAP, Et_3N, DMSO, rt. (i) Fluorescein isothiocyanate, Et_3N, THF, rt.

activation domain to stimulate the transcription of a reporter gene in cells, whereas it had much milder effects on those of the activation domains of VP16 and NF-κB p65, two functionally irrelevant activation domains structurally similar to the ESX activation domain (Figure 2B).[18] In contrast, compound **4d**, which exhibited little activity in killing SK-BR3 cells, had almost no effects on the ESX activation domain (Figure 2B). Western blot analysis of wrenchnolol-treated SK-BR3 cells showed that wrenchnolol (**7b**) reduces the expression of the *Her2* protein in cells but not that of α-tubulin (Figure 2C). Although expression of other genes may be influenced by wrenchnolol, these cellular effects are consistent with its inhibition of the ESX−Sur-2 interaction in vitro.

Synthetically, the tosyl group in wrenchnolol protected the labile O-substituted indole from decomposition under acidic conditions and permitted the convenient synthesis of wrench-

Table 1. Structure–Activity Relationship of Adamanolol Derivatives



Compound	R^1	R^2	R^3	IC_{50} (cell) (μM)	IC_{50} (*in vitro*) (μM)
4a (adamanolol)	1-*H*-indol-4-yl	isopropyl	adamantyl	6.4 ± 1.8	15.4 ± 0.3
4b	1-*H*-indol-4-yl	isobutyl	adamantyl	4.1 ± 0.2	15.1 ± 0.8
4c	1-*H*-indol-4-yl	*tert*-butyl	adamantyl	7.6 ± 1.5	ND
4d	1-*H*-indol-4-yl	H	adamantyl	>100	>40
4e	1-*H*-indol-4-yl	NHBoc	adamantyl	11.7 ± 0.1	11.9 ± 0.7
5a	1-tosyl-1-*H*-indol-4-yl	isopropyl	adamantyl	2.7 ± 0.3	10.0 ± 1.0
5b	1-(8-quinolinesulfonyl)-1-*H*-indol-4-yl	isopropyl	adamantyl	5.0 ± 0.3	12.0 ± 0.9
5c	1-(2-thiophenesulfonyl)-1-*H*-indol-4-yl	isopropyl	adamantyl	6.4 ± 1.5	ND
5d	1-(2-naphthalenesulfonyl)-1-*H*-indol-4-yl	isopropyl	adamantyl	9.7 ± 0.4	ND
5e	1-(biphenyl-4-sulfonyl)-1-*H*-indol-4-yl	isopropyl	adamantyl	5.3 ± 0.2	ND
5f	1-(4-isopropyl-benzenesulfonyl)-1-*H*-indol-4-yl	isopropyl	adamantyl	4.9 ± 0.4	ND
7a	1-tosyl-1-*H*-indol-4-yl	NH_2 • TFA	adamantyl	4.7 ± 0.1	10.2 ± 1.1
7b (wrenchnolol)	1-tosyl-1-*H*-indol-4-yl	NH_2 • TFA	adamantyl	6.9 ± 1.0	10.0 ± 1.5
11a	1-*H*-indol-4-yl	isopropyl	methyl	>100	>40
11b	1-*H*-indol-4-yl	isopropyl	4-phenylphenyl	14.8 ± 1.9	ND
11c	1-*H*-indol-4-yl	isopropyl	2-thenyl	>35	ND
11d	1-*H*-indol-4-yl	isopropyl	*p*-tolyl	>35	ND
12a	methyl	isopropyl	adamantyl	>100	>40
12b	1-naphthyl	isopropyl	adamantyl	10.7 ± 0.6	ND

nolol (**7b**) (Scheme 1). Glycidyl 4-indolyl ether (**1a**) was first tosylated to give tosylate **1b**, which was then coupled with mono-*N*-Boc-1,5-pentanediamine to give amine **2f**. Carbonyldiimidazole-mediated coupling of amine **2f** and amide **3** afforded *N*-Boc-wrenchnolol (**6b**), and subsequent acid treatment of **6b** furnished wrenchnolol (**7b**). This stable and water-soluble derivative of adamanolol can readily be synthesized in a large scale and is capable of receiving further modifications through its extended handle. The handle can be protonated when needed or coupled with a fluorescein group or a biotinylated molecule for mechanistic analysis (see below). Wrenchnolol is a chemically tractable bioactive compound that is suited for mechanistic analysis and for further application.

NMR Analysis of Wrenchnolol with a Water-Soluble Handle. When the amino group of the handle was protonated, wrenchnolol (**7b**) and its aminopropyl version (**7a**) became soluble up to ~1 mM in neutral aqueous solution. The high water solubility enabled NMR analysis of these molecules in a

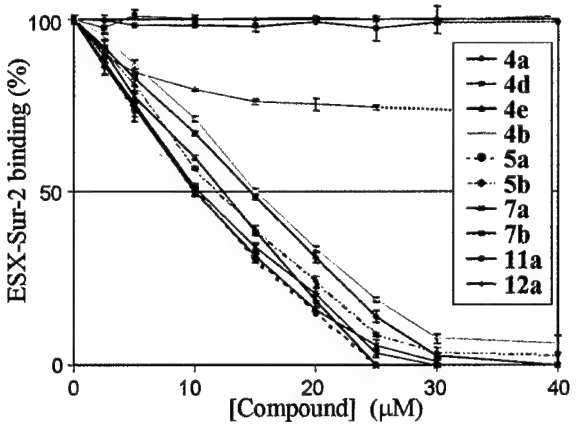
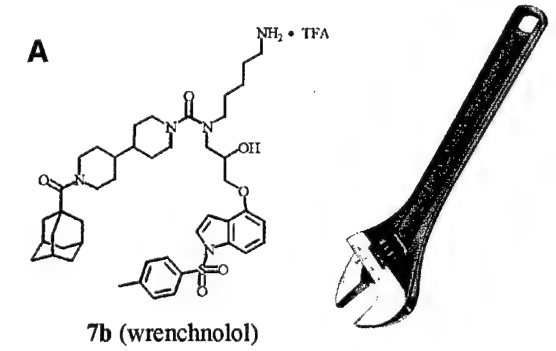
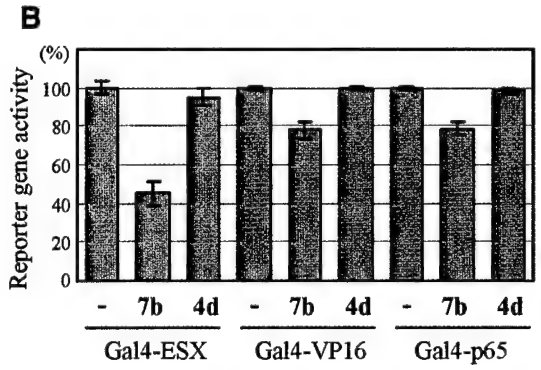


Figure 1. Inhibition of the ESX−Sur-2 interaction by adamanolol derivatives. The binding fractions were calculated on the basis of fluorescence spectra of FITC-$ESX_{129-145}$ (152 nM) in the presence of GST−$Sur2_{352-625}$ (212 nM) and varied concentrations of each adamanolol derivative.





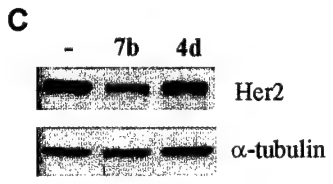


Figure 2. Design and activity of wrenchnolol. (A) The wrench-like structure of wrenchnolol (**7b**). (B) Wrenchnolol (20 μM) selectively impairs the ability of the ESX activation domain to activate the secreted alkaline phosphatase reporter gene in HEK293 cells, whereas **4d** (20 μM) has little effect on the transcriptional activation. (C) Inhibition of *Her2* protein expression by wrenchnolol. SK-BR3 breast cancer cells were treated with wrenchnolol (7 μM) or **4d** (15 μM) for 24 h, and cell lysates were analyzed by western blots.

buffered aqueous solution, and their proton signals were completely assigned through a combination of NOESY, DQF−COSY, and HOHAHA data sets (Table S1). NOESY spectra of **7a** showed clear long-range NOEs between the adamantane

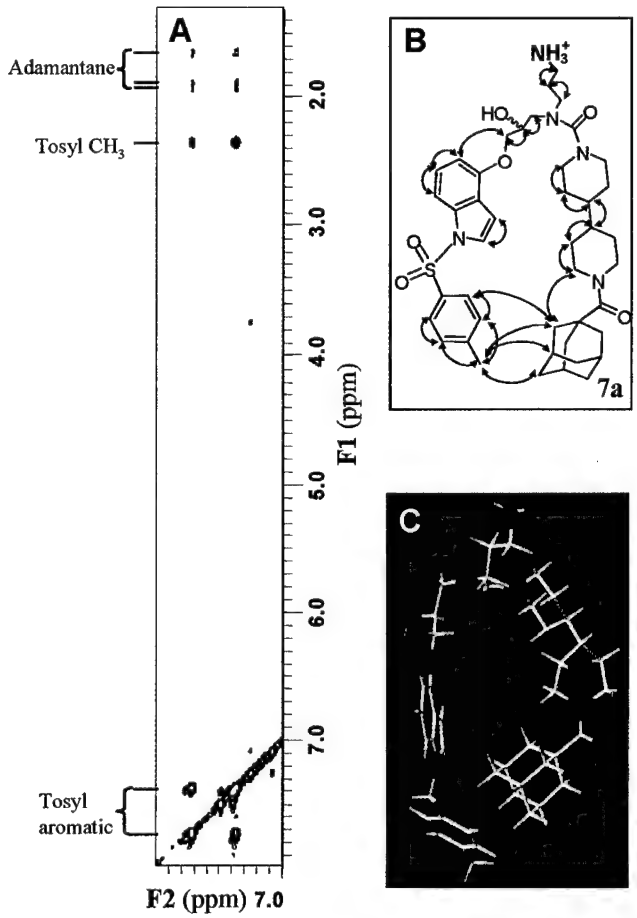


Figure 3. NMR analysis of **7a**. (A) Expanded region of a NOESY spectrum of **7a**. NOE cross-peaks among the aromatic and methyl protons of the tosyl group and the adamantane protons were clearly observed in an aqueous buffered solution (PBS). (B) Chemical structure of **7a** with a summary of key NOE connectivities. (C) Tertiary geometry model of **7a**. A model of the *S* isomer is shown.

protons and the tosyl protons, demonstrating that these two groups are in close proximity in water (Figure 3A, B). DQF−COSY spectra also revealed a highly constrained structure of the arm extended from the indole ring.

Molecular modeling with the NMR constraints supported the s-cis configuration of the urea linker and a wrench-like shape of the molecule (Figure 3C). In the model, the nonpolar components are clustered on one face of the molecule, and the polar animo handle is located away from the hydrophobic face. Although it remains unclear at this point if wrenchnolol binds Sur-2 in the same conformation, the structural characteristics of free wrenchnolol are analogous to those of an amphiphilic α-helical peptide ligand. Wrenchnolol may be a nonpeptidic organic molecule that presents chemical characteristics of an amphiphilic α-helical peptide without mimicking the α-helical backbone.

The NMR sample of wrenchnolol (**7b**) was next titrated with a GST fusion of Sur-2 protein. Selective proton signals of wrenchnolol were lost upon the addition of GST−$Sur2_{352-625}$, whereas titration with the corresponding amounts of GST had little effects on those signals (Figure 4A). The signals arising from adamantane, tosyl, and indole protons were most strongly diminished, while the proton signals of the aminopentyl handle and the bipiperidine linker had little or milder effects upon the addition of GST−$Sur2_{352-625}$ (Figure 4B). Signal losses are

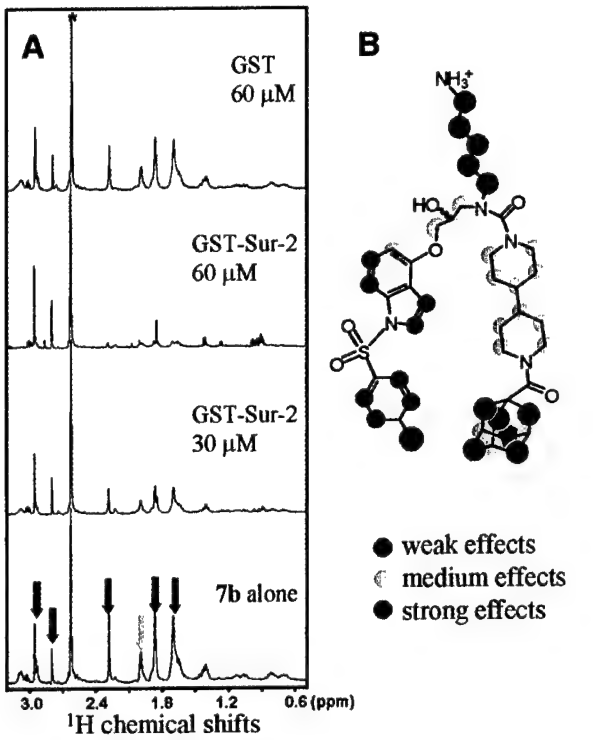


Figure 4. NMR perturbation study of wrenchnolol (**7b**). (A) Expanded one-dimensional ^{1}H NMR spectra of wrenchnolol in the presence or absence of GST−Sur2$_{352-625}$. Addition of 30 or 60 μM of GST−Sur2$_{352-625}$ to the NMR sample of wrenchnolol decreased the signal intensities of the methyl protons of the tosyl group and those of the adamantane protons (red and yellow arrows), whereas the signals of the pentylamine protons had little effect (blue arrows). In contrast, addition of the corresponding amounts of GST (top) had no significant effects on the signals. A star indicates an internal reference signal arising from 10 μL of dimethyl sulfoxide-d_6 (DMSO). (B) A summary of the differential signal intensities. The percentage value of each signal was calculated by using the internal DMSO peak as a reference and classified as a strong (red, < the median), medium (yellow, > the median and < the average), and weak (blue, > the average) effect.

observed when the exchange rate between the free and bound states is intermediate at the NMR time scale and when the chemical shift is perturbed due to the alteration of magnetic environment upon binding. The K_d of wrenchnolol for Sur-2 was estimated to be in a low μM range (see wrenchnolol with a fluorescein handle) where signal losses are often observed in a similar type of interaction. The lost signals arise from the protons located in the jaws of the molecule, supporting the notion that the hydrophobic jaws of amphiphilic wrenchnolol make direct contacts with Sur-2 protein.

Sur-2 Interaction of Wrenchnolol with a Biotinylated Handle. To confirm the interaction of wrenchnolol with Sur-2 in a cellular context, a biotin conjugate of wrenchnolol (**8**) was synthesized (Scheme 1) and incubated with cell nuclear extracts. The bound proteins were purified through an avidin affinity column, separated on an SDS-PAGE gel, and analyzed by western blots with a Sur-2 antibody. As shown in Figure 5A, Sur-2 protein was retained on the affinity column in the presence of biotin conjugate **8**, whereas no Sur-2 protein was detected in its absence or in the presence of a control biotin molecule (**13**). The retention of Sur-2 on the affinity column indicates that wrenchnolol is capable of binding to Sur-2 in the presence of other cellular proteins. Silver-staining detection of all the protein bands on the gel showed a significantly reduced number of protein bands after the affinity purification (compare lanes 2

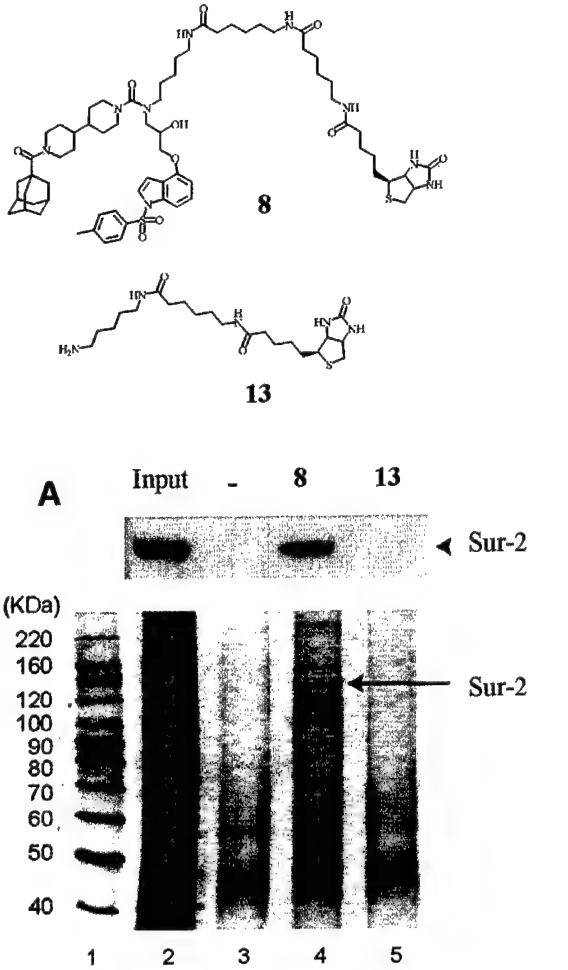


Figure 5. Interaction of biotin-labeled wrenchnolol (**8**) with Sur-2 in cell extracts. Conjugate **8** was incubated with cell nuclear extracts, and bound proteins were purified by an avidin affinity column. (A) Western blot detection of Sur-2 protein. It is evident that Sur-2 was copurified with conjugate **8** (lane 4), whereas no Sur-2 protein was detected with the controls of DMSO (lane 3) and compound **13** (lane 5). (B) Silver staining of the bound proteins. The 130 KDa band indicated by an arrow matched the western-blotted band shown in (A). Lanes 1 and 2 show protein markers and 10% input of nuclear extracts, respectively.

and 4 in Figure 5B), demonstrating some degree of binding selectivity of wrenchnolol. One of the silver-stained bands matched the western-blotted band of Sur-2, while the identities of the other bands remained unknown. Sur-2 (also called DRIP130 or CRSP130) is a subunit of the human mediator complexes:[23−26] The additional bands may include those of the other subunits copurified with Sur-2. However, all the protein bands cannot be explained by the subunits of human mediator complexes or Sur-2-interacting proteins, suggesting that the selectivity of wrenchnolol remains to be further optimized.

Wrenchnolol with a Fluorescein Handle. A synthetic compound with a molecular weight higher than 500 is generally less likely to penetrate the cell membrane. To check the cell permeability of wrenchnolol, whose molecular weight is 802, its handle was coupled with a fluorescein molecule through a

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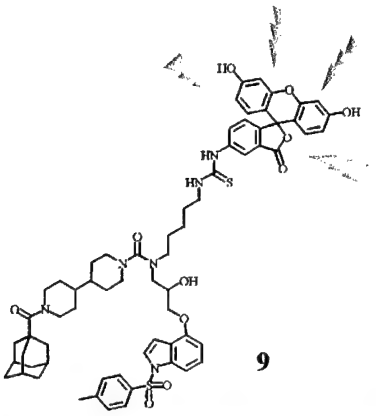

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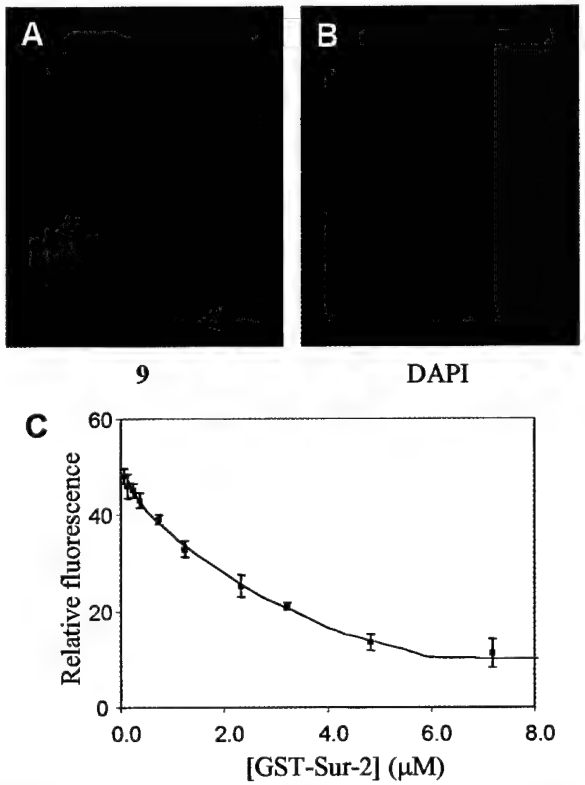

Figure 6. Wrenchnolol with a fluorescein handle (**9**). (A) Evaluation of cell permeability of wrenchnolol. Conjugate **9** was incubated with SK-BR3 cells for 1 h, and fluorescence images were captured by a fluorescence microscope. (B) Costaining of the cells with nucleus-specific DAPI dye. (C) Estimation of K_d of the wrenchnolol−Sur-2 interaction. Fluorescence spectra of conjugate **9** were monitored upon addition of GST−$Sur2_{352-625}$. Shown are relative emission intensities of **9** at 522 nm (excitation: 490 nm) in the presence of varied concentrations of GST−$Sur2_{352-625}$.

simple thiourea formation (Scheme 1). As shown in Figure 6A, fluorescein conjugate **9** crossed the cell membrane of breast cancer cells and diffused both in the cytoplasm and the nucleus. However, careful comparison of the fluorescence images of fluorescein conjugate **9** (Figure 6A) and the nucleus-specific DAPI dye (Figure 6B) revealed that the compound is located more preferentially in the cytoplasm than in the nucleus, as often observed with large synthetic organic molecules.[27] Although wrenchnolol is capable of penetrating the cell membrane, improvement of its nuclear localization might strengthen the biological activity and selectivity.

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The fluorescein conjugate **9** also enabled evaluation of the K_d value of the interaction between wrenchnolol and Sur-2 in vitro. When a GST fusion of Sur-2 protein (GST−$Sur\text{-}2_{352-625}$) was added to a solution of conjugate **9**, the fluorescence intensity of **9** at 522 nm was decreased (Figure 6C). The clear fluorescence perturbation by the interaction permitted an estimation of the K_d value to be 1.8 μM, which is consistent with the IC_{50} of wrenchnolol in cells.

Discussion

Inhibition of a Nuclear Protein−Protein Interaction by Small Molecules. Small molecules that target a protein−protein interaction in the nucleus need to penetrate the cell membrane, diffuse into the nucleus, and bind selectively to a protein target with a consequent cellular phenotype. Development of such molecules is challenging and may require tremendous efforts before clinical application. Nevertheless, given the importance of the problem in enriching future opportunities, efforts in this direction need to continue.[28,29] The case study of wrenchnolol foreshadows the promise and the challenge of targeting a protein−protein interaction in the nucleus.

Recent evidence has suggested that protein interactions that are mediated by short α-helical peptides are tractable to inhibition by small synthetic molecules. For example, the interaction of Bcl-X_L with Bak, a well-documented association that controls programmed cell death, is mediated by a short α-helical peptide of Bak and can be inhibited by nonpeptidic small molecules.[30−33] However, inhibitors of protein−protein interactions in general tend to be highly hydrophobic due to the nonpolar nature of binding pockets and may lack the water solubility required for structural or animal studies. Our results suggest that designing a tetrasubstituted urea molecule is a potential approach to obtaining a water-soluble inhibitor of α-helix-mediated protein interactions. The bulky amino handle of wrenchnolol enforced the active conformation and rendered it water-soluble enough to permit NMR characterization in an aqueous solution and its evaluation in mouse models of breast cancers (currently underway). A number of bioactive peptide ligands are amphiphilic helices with a hydrophilic face on the opposite side of the hydrophobic binding interface. The wrench-shape design of urea molecules could be an effective way of mimicking an amphiphilic α-helical ligand by synthetic molecules.

Our results suggest that the s-cis conformation of adamanolol is an important determinant of the activity. The preorganized hydrophobic surface of adamanolol is perhaps complementary to the pocket in Sur-2, and its modification should influence the activity. We synthesized two adamanolol analogues having distinct jaw widths (Chart 2):[34] in one molecule, the bipiperidine linker was extended by inserting a benzene ring (**14**); in the other, the bipiperidine linker was replaced by a shorter pipera-

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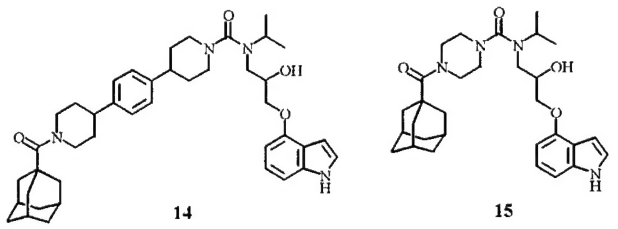
Chart 2



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dine (**15**). Their evaluation in SK-BR3 cells showed that either the extension or shortening reduced the activity of adamanolol, suggesting that the bipiperidine linker of adamanolol intrinsically has a perfect length for binding to Sur-2. Diversification of the width and shape in the jaws of adamanolol or wrenchnolol may lead to the discovery of inhibitors of distinct α-helix-mediated protein interactions.

Despite the benefits, the wrench-shape design has a drawback: the relatively large size of the molecules, which might limit their cell permeability. This problem may be intrinsic to any competitive inhibitors of protein−protein interactions, which, unlike enzyme inhibitors, need to cover a larger binding interface to achieve selectivity and potency. Although our analysis showed that wrenchnolol is capable of penetrating the cell membrane and diffusing both in the cytoplasm and in the nucleus, its nuclear distribution is somewhat limited, as often observed with large synthetic molecules.[27] Nuclear pores are considered to be large enough for even 10 KDa proteins to cross passively;[35] however, there may be an active transport mechanism to eliminate certain types of synthetic molecules from the nucleus. Cellular distribution of small-molecule inhibitors, especially protein−protein interaction inhibitors, is perhaps a key determinant of their biological effectiveness, just as the subcellular localization of signaling proteins plays an essential role in achieving their specific interactions with the downstream effectors. An understanding of the molecular characteristics that govern cellular localization of large synthetic molecules would accelerate the success of the inhibitors of protein−protein interactions.

Transcription Modulators. The regulation of transcription often causes drastic phenotypic changes in human bodies through its effects on differentiation, organ development, aging, and diseases. External manipulation of transcription by cell-permeable synthetic molecules represents a challenge in medicinal and bioorganic chemistry.[4,36−43] A large number of studies on naturally occurring transcription factors have established the dogma that they typically have separable domains for sequence-specific binding to DNA and for transcriptional activation through protein−protein interactions.[5,6] Efforts to incorporate synthetic counterparts of these functional modules have been made with the goal of developing a small-molecule transcription factor. Dervan and co-workers have shown that the DNA-binding module of a transcription factor can be replaced by a hairpin polyamide composed of *N*-methylpyrrole and *N*-methylimidazole amino acids that binds in the minor groove of DNA.[38−40] This cell-permeable polyamide is capable of targeting a predetermined DNA sequence with affinities and specificities comparable to transcription factors.[44−46] Although these studies provided a key initial step toward the goal of engineering cell-permeable small-molecule transcription factors, the use of peptides as an activation module prevented the artificial transcription factors from crossing the cell membrane and limited their application. A goal of the field is now the replacement of the activation peptide with nonpeptidic organic molecules in order to engineer cell-permeable compounds.

Wrenchnolol is a cell-permeable synthetic molecule that binds to the Sur-2 subunit of the human mediator complex by mimicking the potent activation domain of ESX. It is also chemically tractable and capable of receiving further modifications through its handle. Wrenchnolol may serve as a nonpeptidic activation module and could lead to the development of synthetic modulators of gene transcription.

Experimental Section

General Procedures. SK-BR3 cells were kindly provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center) and grown in DMEM/F-12 medium with 10% FBS (Invitrogen). GST−$Sur2_{352-625}$ and FITC-$ESX_{129-145}$ were prepared and characterized as described before.[18] Compounds **11c,d** and **13** were purchased from Tripos and Sigma, respectively. The solvents used for chemical synthesis were dried prior to use. All other chemicals were used as received without purification. All moisture-sensitive reactions were performed in flame-dried and/or oven-dried glassware under a positive pressure of nitrogen unless otherwise noted. Thin-layer chromatography was carried out with glass TLC plates precoated with Merck silica gel 60 F254. Column chromatography was accomplished with Fuji Silysia Chemical silica gel BW820MH. Proton nuclear magnetic resonance spectra were recorded in deuterated solvents at 270 or 600 MHz. High-resolution mass spectra were obtained by Q-star spectrometer (Applied Biosystems) on an ESI mode.

Glycidyl 4-Indolyl Ether (1a). To a solution of 4-hydroxyindole (0.50 g, 3.8 mmol) in dimethyl sulfoxide (10 mL) at 45 °C were added potassium hydroxide (0.43 g, 7.6 mmol) and then epichlorohydrin (1.4 g, 15.2 mmol). After being stirred at 45 °C for 4 h, the reaction mixture was diluted with a saturated solution of NH_4Cl and extracted with diethyl ether (3 × 50 mL). The combined organic layers were dried with Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with benzene/acetone mixtures to give the glycidyl 4-indolyl ether (**1a**) (0.75 g, quant) as a colorless oil: IR (thin film) 3402, 2923, 1497, 1362, 1244 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 8.17 (br. s, 1H), 7.14−7.03 (m, 3H), 6.70 (t, J = 2.0 Hz, 1H), 6.62 (dd, J = 1.1, 7.0 Hz, 1H), 4.35 (dd, J = 3.5, 11.1 Hz, 1H), 4.16 (dd, J = 5.4, 11.1 Hz, 1H), 3.46 (m, 1H), 2.94 (dd, J = 4.1, 4.9, 1H), 2.82 (dd, J = 2.7, 4.9, 1H); HRMS (ESI) exact mass calcd for $C_{11}H_{11}NO_2$ + H requires *m/z* 190.0868, found *m/z* 190.0842.

Preparation of Glycidyl *N*-Tosyl-4-indolyl Ether (1b). A solution of glycidyl 4-indolyl ether (**1a**) (1.0 g, 5.3 mmol) in THF (5 mL) was cooled to 0 °C, and sodium hydride in mineral oil (60%, 0.42 g, 10.6 mmol) and tosyl chloride (1.2 g, 6.3 mmol) were added. After being

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stirred at this temperature for 1 h, the reaction mixture was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with water and extracted with $CHCl_3$ (3 × 50 mL). The combined organic layers were dried with Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with benzene/acetone mixtures to give the glycidyl *N*-tosyl-4-indolyl ether (**1b**) (1.5 g, 82%) as a white solid: IR (thin film) 2930, 1362, 1189, 1137, cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.75 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.23 (t, J = 8.4 Hz, 1H), 7.21 (d, J = 8.4 Hz, 2H), 6.80 (d, J = 3.8 Hz, 1H), 6.63 (d, J = 8.4 Hz, 1H), 4.32 (dd, J = 3.2, 11.1 Hz, 1H), 4.03 (dd, J = 5.7, 11.1 Hz, 1H), 3.38 (m, 1H), 2.91 (t, J = 4.9 Hz, 1H), 2.77 (dd, J = 2.7, 4.9 Hz, 1H), 2.34 (s, 3H); HRMS (ESI) exact mass calcd for $C_{18}H_{17}NO_4S$ + H requires *m/z* 344.0957, found *m/z* 344.0944.

***N*-[3-(4-Indolyloxy)-2-hydroxypropyl]-isobutylamine (2a).** To a solution of glycidyl 4-indolyl ether (**1a**) (215 mg, 1.1 mmol) in methanol (2 mL) was added isobutylamine (160 mg, 2.2 mmol). The solution was heated to 100 °C and stirred for 32 h. The product was purified by column chromatography on silica gel with $CHCl_3$/methanol mixtures to give the corresponding amine (**2a**) (120 mg, 42%) as a colorless oil: IR (thin film) 3404, 2954, 1508, 1363 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 8.24 (br. s, 1H), 7.12−7.02 (m, 3H), 6.65 (t, J = 2.4 Hz, 1H), 6.51 (dd, J = 1.0, 7.3 Hz, 1H), 4.30 (m, 1H), 4.21−4.09 (m, 2H), 3.43 (br. s, 2H), 3.05 (dd, J = 3.8, 12.4 Hz, 1H), 2.96 (dd, J = 7.8, 12.4 Hz, 1H), 2.61 (dd, J = 2.7, 11.9 Hz, 1H), 2.57 (dd, J = 2.7, 11.9 Hz, 1H), 1.90 (m, 1H), 0.97 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.8 Hz, 3H); HRMS (ESI) exact mass calcd for $C_{15}H_{22}N_2O_2$ + H requires *m/z* 263.1760, found *m/z* 263.1778.

***N*-[3-(*N*-Tosyl-4-indolyloxy)-2-hydroxypropyl]-*N'*-Boc-1,5-pentanediamine (2f).** The reaction of glycidyl *N*-tosyl-4-indolyl ether (**1b**) (1.7 g, 5.0 mmol) and mono-*N*-Boc-1,5-pentanediamine (2.0 g, 10 mmol) as described for **2a** gave the title compound (**2f**) (1.7 g, 63%) as a colorless oil: IR (thin film) 3426, 2932, 1684, 1508, 1364, 1160 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.74 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.4 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.20 (d, J = 8.4 Hz, 2H), 7.19 (t, J = 8.4 Hz, 1H), 6.76 (d, J = 3.8 Hz, 1H), 6.64 (d, J = 8.4 Hz, 1H), 4.54 (s, 1H), 4.07 (m, 3H), 3.10 (br. m, 2H), 2.89 (dd, J = 3.5, 12.4 Hz, 1H), 2.78 (dd, J = 7.0, 12.4 Hz, 1H), 2.65 (dt, J = 2.4, 4.3 Hz, 2H), 2.33 (s, 3H), 2.08 (br. s, 2H), 1.60−1.28 (m, 6H), 1.44 (s, 9H); HRMS (ESI) exact mass calcd for $C_{28}H_{39}N_3O_6S$ + H requires *m/z* 546.2638, found *m/z* 546.2640.

Preparation of Amide 3. To a solution of 4,4′-bipiperidine (0.84 g, 5.0 mmol) and triethylamine (2 mL) in $CHCl_3$ (150 mL) was added a solution of 1-adamantanecarbonyl chloride (0.50 g, 2.5 mmol) in $CHCl_3$ (50 mL). The resulting mixture was refluxed for 0.5 h, diluted with water, and extracted with $CHCl_3$ (3 × 100 mL). The combined extracts were dried over Na_2SO_4 and then concentrated in a vacuum. The residue was purified by column chromatography on silica gel with methanol/aqueous ammonia mixtures to give amide **3** (750 mg, 90%) as a colorless oil: IR (thin film) 3421, 2902, 1617 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 4.46 (br. d, J = 13.0 Hz, 2H), 3.02 (br. d, J = 11.6 Hz, 2H), 2.91 (br. s, 1H), 2.59 (br. t, J = 11.9 Hz, 2H), 2.47 (br. t, J = 11.9 Hz, 2H), 1.95 (br. s, 3H), 1.90 (br. s, 6H), 1.63 (br. s, 10H), 1.19−1.02 (br. m, 6H); HRMS (ESI) exact mass calcd for $C_{21}H_{34}N_2O$ + H requires *m/z* 331.2749, found *m/z* 331.2753.

Preparation of Adamanolol (4a). To a solution of amide **3** (120 mg, 0.36 mmol) in THF (10 mL) was added 1,1′-carbonyldiimidazole (240 mg, 1.46 mmol). After being refluxed for 4 h, the mixture was diluted with $CHCl_3$ (20 mL) and washed with water (3 × 20 mL). The organic layer was dried with Na_2SO_4 and concentrated to give carbamoyl imidazole, which was used without further purification. A solution of carbamoyl imidazole and MeI (0.5 mL) in MeCN (2 mL) was stirred for 18 h at room temperature. Evaporation of the solvent and excess MeI in a vacuum gave the corresponding imidazolium salt. To a solution of imidazolium salt in CH_2Cl_2 (2 mL) were added pindolol (268 mg, 1.08 mmol) and Et_3N (0.5 mL). After being stirred at room temperature for 30 h, the mixture was concentrated and purified by column chromatography on silica gel with $CHCl_3$/methanol mixtures to give adamanolol (**4a**) (145 mg, 67%) as a colorless oil: IR (thin film) 3450, 2907, 1624, 1428, 1245 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 8.57 (br. s, 1H), 7.11−7.04 (m, 3H), 6.59 (t, J = 2.4 Hz, 1H), 6.52 (dd, J = 2.7, 5.7 Hz, 1H), 5.29 (br. m, 1H), 4.53 (br. d, J = 13.5 Hz, 2H), 4.45−4.25 (m, 2H), 4.12 (br. m, 2H), 3.44 (dd, J = 1.4, 13.5 Hz, 1H), 3.35−3.10 (m, 2H), 2.82−2.50 (br. m, 4H), 2.03 (br. s, 3H), 1.99 (br. s, 6H), 1.72 (s, 6H), 1.80−1.44 (br. m, 4H), 1.35 (d, J = 6.5 Hz, 3H), 1.32 (d, J = 6.5 Hz, 3H), 1.45−0.86 (m, 6H); HRMS (ESI) exact mass calcd for $C_{36}H_{52}N_4O_4$ + H requires *m/z* 605.4067, found *m/z* 605.4065.

Preparation of *N*-Tosyladamanolol (5a). A solution of adamanolol (**4a**) (30.1 mg, 0.05 mmol) in THF (1 mL) was cooled to 0 °C, and sodium hydride in mineral oil (60%, 12 mg, 0.50 mmol) and tosyl chloride (9.5 mg, 0.05 mmol) were added. After being stirred at this temperature for 0.5 h, the reaction mixture was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with brine and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried with Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with $CHCl_3$/methanol mixtures to give the *N*-tosyladamanolol (**5a**) (21.5 mg, 57%) as a colorless oil: IR (thin film) 3434, 2910, 2852, 1623, 1427, 1188 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.75 (d, J = 8.4 Hz, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.45 (d, J = 3.5 Hz, 1H), 7.21 (d, J = 8.4 Hz, 2H), 7.20 (dd, J = 7.8, 8.4 Hz, 1H), 6.75 (d, J = 3.5 Hz, 1H), 6.68 (d, J = 7.8 Hz, 1H), 5.14 (br. m, 1H), 4.55 (br. d, J = 13.0 Hz, 2H), 4.24 (br. d, J = 4.6 Hz, 2H), 4.32−3.94 (br. m, 2H), 2.96 (m, 2H), 2.82 (m, 1H), 2.75−2.54 (br. m, 4H), 2.34 (s, 3H), 2.03 (br. s, 3H), 1.99 (br. s, 6H), 1.72 (s, 6H), 1.80−1.44 (br. m, 4H), 1.45−0.86 (m, 6H), 1.04 (d, J = 6.5 Hz, 6H); HRMS (ESI) exact mass calcd for $C_{43}H_{58}N_4O_6S$ + H requires *m/z* 759.4155, found *m/z* 759.4138.

Preparation of *N*-Boc-Wrenchnolol with an Aminopropyl Handle (6a). The reaction of *N*-[3-(*N*-tosyl-4-indolyloxy)-2-hydroxypropyl]-*N'*-Boc-1,3-propanediamine (**2e**) (460 mg, 0.89 mmol) and monoamide **3** (250 mg, 0.76 mmol) as described for **4a** gave the title compound (**6a**) (353 mg, 53%) as a colorless oil: IR (thin film) 3441, 1633 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.75 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.4 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.26−7.17 (m, 3H), 6.74 (d, J = 3.8 Hz, 1H), 6.67 (d, J = 8.4 Hz, 1H), 5.26 (br. m, 1H), 5.10 (br. s, 1H), 4.55 (br. d, J = 13.0 Hz, 2H), 4.29 (br. m, 2H), 4.30−3.85 (br. m, 2H), 3.25−3.05 (br. m, 2H), 2.95−2.78 (br. m, 2H), 2.78−2.55 (br. m, 6H), 2.34 (s, 3H), 2.03 (br. s, 3H), 1.99 (br. s, 6H), 1.90−1.55 (br. m, 6H), 1.72 (s, 6H), 1.40 (s, 9H), 1.30−0.70 (br. m, 6H); HRMS (ESI) exact mass calcd for $C_{48}H_{67}N_5O_8S$ + H requires *m/z* 874.4789, found *m/z* 874.4778.

Preparation of *N*-Boc-Wrenchnolol (6b). The reaction of *N*-[3-(*N*-tosyl-4-indolyloxy)-2-hydroxypropyl]-*N'*-Boc-1,5-pentanediamine (**2f**) (500 mg, 0.92 mmol) and monoamide **3** (250 mg, 0.76 mmol) as described for **4a** gave the title compound (**6b**) (322 mg, 47%) as a colorless oil: IR (thin film) 3445, 2933, 1684, 1617, 1428 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.75 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 7.8 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.21 (d, J = 8.4 Hz, 2H), 7.26−7.19 (t, J = 7.8 Hz, 1H), 6.74 (d, J = 3.8 Hz, 1H), 6.67 (d, J = 7.8 Hz, 1H), 5.18 (br. m, 1H), 4.55 (br. m, 1H), 4.55 (br. d, J = 12.7 Hz, 2H), 4.24 (br. d, J = 4.6 Hz, 2H), 4.32−3.87 (br. m, 2H), 3.17−2.91 (m, 4H), 2.79−2.52 (m, 6H), 2.34 (s, 3H), 2.03 (br. s, 3H), 1.99 (br. s, 6H), 1.72 (s, 6H), 1.44 (s, 9H), 1.80−0.86 (m, 16H); HRMS (ESI) exact mass calcd for $C_{50}H_{71}N_5O_8S$ + H requires *m/z* 902.5102, found *m/z* 902.5100.

Wrenchnolol with an Aminopropyl Handle (7a). To a solution of **6a** (350 mg, 0.40 mmol) in $CHCl_3$ (3 mL) was added TFA. This solution was stirred at room temperature for 15 min and then concentrated in a vacuum, which was used without further purification to give **7a** (360 mg, quant) as a colorless oil: IR (thin film) 3436, 1630 cm^{-1}; ^1H NMR ($CDCl_3$−CD_3OD/4:1, 270 MHz) δ_H 7.62 (d, J = 8.4 Hz, 2H), 7.47 (d,

J = 8.1 Hz, 1H), 7.35 (d, J = 3.8 Hz, 1H), 7.24 (t, J = 8.1 Hz, 1H), 7.11 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 3.8 Hz, 1H), 6.56 (d, J = 8.1 Hz, 1H), 5.24 (br. m, 1H), 4.40 (br. d, J = 11.9 Hz, 2H), 4.17 (br. m, 2H), 4.05−3.72 (br. m, 2H), 3.31 (br. m, 2H), 3.04 (br. m, 2H), 2.91 (br. m, 2H), 2.78−2.42 (br. m, 4H), 2.22 (s, 3H), 2.05 (br. m, 2H), 1.91 (br. s, 3H), 1.85 (br. s, 6H), 1.70−1.30 (br. m, 4H), 1.57 (s, 6H), 1.30−0.60 (br. m, 6H); HRMS (ESI) exact mass calcd for $C_{43}H_{59}N_5O_6S$ + H requires m/z 774.4264, found m/z 774.4286.

Preparation of Wrenchnolol (7b). The reaction of *N*-Boc-wrenchnolol (**6b**) (320 mg, 0.36 mmol) as described for **7a** gave the title compound (**7b**) (325 mg, quant) as a colorless oil: IR (thin film) 3444, 2910, 1635, 1428 cm^{-1}; ^1H NMR ($CDCl_3$, 270 MHz) δ_H 7.75 (d, J = 8.1 Hz, 2H), 7.58 (d, J = 8.1 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.21 (d, J = 8.1 Hz, 2H), 7.13−7.02 (dd, J = 7.8, 8.1 Hz, 1H), 6.74 (d, J = 3.8 Hz, 1H), 6.65 (d, J = 7.8 Hz, 1H), 5.30 (br. m, 1H), 4.54 (br. m, 1H), 4.54 (br. d, J = 12.7 Hz, 2H), 4.24 (br. d, J = 4.3 Hz, 2H), 4.32−3.81 (br. m, 2H), 3.08 (br. d, J = 5.4 Hz, 1H), 2.85 (br. t, J = 5.4 Hz, 1H), 2.81−2.52 (br. m, 8H), 2.34 (s, 3H), 1.99 (br. s, 9H), 1.72 (br. s, 6H), 1.79−0.70 (m, 16H); HRMS (ESI) exact mass calcd for $C_{45}H_{63}N_5O_6S$ + H requires m/z 802.4577, found m/z 802.4550.

Biotin Conjugate Wrenchnolol (8). To a solution of wrenchnolol (**7b**) (5.0 mg, 0.006 mmol) and triethylamine (0.1 mL) in DMSO (0.25 mL) were added 4-(dimethylamino)pyridine (0.1 mg), *N,N'*-dicyclohexylcarbodiimide (2.5 mg, 0.012 mmol), and biotoin-XX-NHS (6.8 mg, 0.012 mmol). This solution was stirred at room temperature overnight, diluted with brine, and extracted with $CHCl_3$ (3 × 10 mL). The combined extracts were dried over Na_2SO_4 and then concentrated in a vacuum. The residue was purified by column chromatography on silica gel with $CHCl_3$/methanol mixtures to give the corresponding amide (**8**) (7.2 mg, 95%) as a colorless oil: IR (thin film) 3284, 2933, 1684, 1653, 1559, 1541 cm^{-1}; ^1H NMR (CD_3OD, 270 MHz) δ_H 7.79 (d, J = 8.6 Hz, 2H), 7.60 (d, J = 8.1 Hz, 1H), 7.56 (d, J = 4.0 Hz, 1H), 7.31 (d, J = 8.6 Hz, 2H), 7.21 (t, J = 8.1 Hz, 1H), 6.79 (d, J = 4.0 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 5.38 (br. m, 1H), 4.60−4.42 (br. m, 3H), 4.38−4.20 (br. m, 3H), 4.16−3.89 (br. m, 2H), 3.47 (br. m, 2H), 3.24−3.01 (br. m, 9H), 2.91 (dd, J = 5.0, 12.8 Hz, 1H), 2.85−2.54 (br. m, 5H), 2.34 (s, 3H), 2.24−2.08 (br. m, 6H), 2.00 (br. s, 9H), 1.77 (br. s, 6H), 1.90−0.40 (m, 34H); HRMS (ESI) exact mass calcd for $C_{67}H_{99}N_9O_{10}S_2$ + H requires m/z 1254.7035, found m/z 1254.7010.

Fluorescein Conjugate Wrenchnolol (9). To a solution of wrenchnolol (**7b**) (10 mg, 0.012 mmol) and triethylamine (0.2 mL) in THF (1 mL) was added fluorescein isothiocyanate (5.6 mg, 0.014 mmol). This solution was stirred at room temperature for 8 h and then concentrated in a vacuum. The residue was purified by column chromatography on silica gel ($CHCl_3$/methanol) and ODS silica gel (70%MeOH, 0.1%TFA) to give the fluorescein conjugate (**9**) (8.3 mg, 58%) as a yellow solid: IR (thin film) 3421, 2910, 1636 cm^{-1}; ^1H NMR (CD_3OD, 270 MHz) δ_H 8.31 (br. s, 0.6H), 8.19 (br. s, 0.4H), 7.83 (m, 1H), 7.73 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.25 (d, J = 8.4 Hz, 2H), 7.38−6.57 (m, 10H), 5.55 (br. m, 1H), 4.45 (br. d, J = 12.7 Hz, 2H), 4.40−4.20 (br. m, 2H), 4.20−3.91 (br. m, 4H), 3.91−3.52 (br. m, 2H), 3.40−3.20 (br. m, 2H), 2.81−2.47 (br. m, 4H), 2.29 (s, 3H), 1.95 (br. s, 9H), 1.72 (br. s, 6H), 1.67−0.45 (m, 16H); HRMS (ESI) exact mass calcd for $C_{66}H_{74}N_6O_{11}S_2$ + H requires m/z 1191.4935, found m/z 1191.4939.

Cell Viability Assays. SK-BR3 breast cancer cells were plated on 96-well plates at a density of 5 × 10^3 cells/well and maintained for 24 h. The cells were then incubated with varied concentrations of each compound for 48 h, followed by addition of Premix WST-1 Solution (Takara). Cell viability was estimated by measuring absorbance at 450 nm after 1 h with a SPECTRAmax microplate reader.

Transcription Reporter Gene Assay. We used the mammalian expression vectors each encoding the activation domain of ESX, VP16, or NF-κB p65 fused with the GAL4 DNA-binding domain (amino acids 1−94) as described. Each expression construct was cotransfected to HEK293Tag human kidney cells together with a reporter plasmid in which the production of secreted alkaline phosphatase (SEAP) is under control of an IL2 promoter carrying five GAL4 binding sites. After 10 h of incubation with wrenchnolol (20 μM), an aliquot of the culture was assayed for SEAP activity as described.[42]

Effects of Wrenchnolol on the Expression Levels of *Her2*. SK-BR3 cells were treated by 7 μM of wrenchnolol for 24 h, and whole cell lysates were analyzed by western blots. The primary antibodies used for the western analysis were a monoclonal antibody against *Her2* (c-neu-Ab-3; Oncogene Science) and a monoclonal antibody against α-tublin (Molecular Probes). The blotted membranes were then visualized with ECL chemiluminescence detection reagents (Amersham Bioscience).

Sur-2 Interaction of a Biotin Conjugate of Wrenchnolol (8). HeLa cell nuclear extracts were prepared as described[47] and incubated with a biotin conjugate of wrenchnolol (**8**) (100 μM) at 4 °C for 24 h. The samples were incubated with Neutravidin affinity resin (Pierce) at 4 °C for 2 h and extensively washed with PBS buffer containing 0.5% Nonidet P-40. The bound proteins were then separated by SDS/PAGE and analyzed by western blots with an antibody against human Sur-2 or by silver-staining.

Fluorescence Studies. To monitor the ability of compounds to inhibit ESX−Sur-2 interaction, fluorescence emission spectra of fluorescein-isothiocyanate-labeled $ESX_{129-145}$ (FITC-$ESX_{129-145}$, 152 nM, excitation at 490 nm) were measured in the presence of GST−$Sur2_{352-625}$ (212 nM) and varied concentrations of a compound. Fluorescence intensity changes at 522 nm as a function of chemical concentrations (0 to 50 μM) were monitored. IC_{50} values were calculated by curve fitting of the data. For shallow dose−effect curves, IC_{50} values could not be accurately determined and were assigned with > marks. For the evaluation of K_d of wrenchnolol, a concentrated solution of GST−$Sur2_{352-625}$ was added to a 534 nM solution of a fluorescein conjugate of wrenchnolol (**9**), and the fluorescence intensity of **9** at 522 nm was monitored. All the measurements were performed in triplicate in a PBS solution containing 100 mM NaCl (pH 7.0) on a Perkin-Elmer LS-50B fluorometer. The K_d of **9** was calculated by curve fitting of the fluorescence intensity as a function of the GST−$Sur2_{352-625}$ concentration, using the following equation:

$$A = A_o + 0.5\Delta\epsilon([\mathbf{9}]_o + (([\mathbf{9}]_o + [P]_o K_d)^2 - 4[\mathbf{9}]_o[P]_o)^{0.5})$$

where A and A_o are the values of fluorescence intensity of **9** in the presence or absence of GST−$Sur2_{352-625}$, respectively, and $\Delta\epsilon$ is the differential fluorescence intensity of 1 μM of **9** in the presence of an infinite concentration of protein and in its absence. $[\mathbf{9}]_o$ and $[P]_o$ are the total concentrations of **9** and protein added, respectively.

NMR Studies. Wrenchnolol (**7b**) or its aminopropyl analogue (**7a**) (0.5 mg) was dissolved in 0.5 mL of a phosphate-buffered saline (PBS) solution (99.99% D_2O, pD = 6.8). Complete assignments of the proton signals were achieved by a combination of NOESY, DQF-COSY, and HOHAHA experiments (Table S1). A model structure of **7a** was acquired using energy minimization with CVFF force field in the Insight 2000 program (Accelrys) and adjusted on the basis of NOE connectivities and J coupling constants. A concentrated PBS solution of GST−$Sur2_{352-625}$ was gradually added to an NMR sample of wrenchnolol (**7b**), and a one-dimensional ^1H spectrum was collected after each addition of the protein. NMR experiments were performed on a Bruker Avance 600 MHz spectrometer. The data were processed using FELIX (Accelrys) and UXNMR (Bruker), and proton chemical shifts were referenced to the HOD resonance (4.70 ppm at 298 K; temperature correction factor: −0.0109 ppm/K). All one-dimensional ^1H NMR spectra were recorded with 12 ppm spectral width, 8192 data points, and 298 K temperature. Suppression of the HOD resonance was achieved by an excitation sculpting gradient pulse. Two-dimensional experiments of DQF-COSY, HOHAHA (88 ms mixing time), and NOESY (150

(47) Dignam, J. D.; Martin, P. L.; Shastry, B. S.; Roeder, R. G. *Methods Enzymol.* **1983**, *101*, 582−598.

and 300 ms mixing times) were recorded with 4096 data points in the t_2 dimension and 512 increments. A 90° phase-shifted sine bell function was used in the t_2 and t_1 dimensions prior to Fourier transformation of NOESY, while a 30° phase-shifted skewed sine bell function was used for HOHAHA and DQF-COSY. The final spectral matrices consisted of 1024 × 1 024 of the ^{1}H frequency (F2 and F1) dimensions.

Cellular Distribution of Wrenchnolol. SK-BR3 cells grown on cover slips were treated with 10 μM of FITC-conjugate **9** for 1 h. After being washed with ice-cold PBS twice, the cells were fixed with 100% methanol at −20 °C for 20 min. The cells were co-stained with DAPI. Fluorescence images of the cells were captured by a Zeiss Axiovert 200M fluorescence microscope.

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Supporting Information Available: Details of the synthetic procedures and NMR data (Table S1 and Figures S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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